Book of Abstracts

9th Conference of the European Foundation for Plant Pathology

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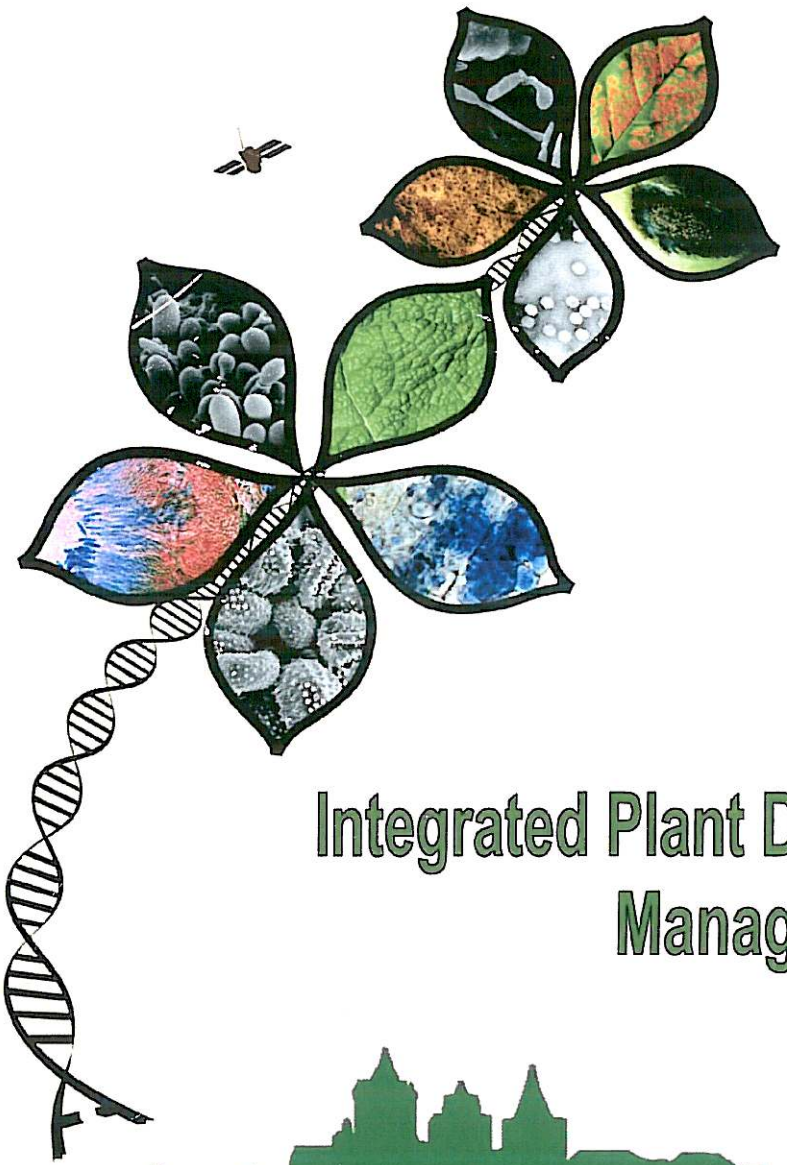

Integrated Plant Disease Management

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9TH CONFERENCE OF THE EUROPEAN FOUNDATION FOR PLANT PATHOLOGY AND 6TH CONGRESS OF THE SOCIEDADE PORTUGUESA DE FITOPATOLOGIA

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Expression analysis by RT-qPCR of *NPP1* gene from *Phytophthora cinnamomi*

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INTRODUCTION

In recent years, a novel class of necrosis-inducing proteins, known as Nep1-like proteins (NLPs) has been identified in bacteria, fungi and oomycetes. Species of the genus *Phytophthora* are causal agents of serious plant diseases infecting *Castanea sativa*. *Phytophthora cinnamomi* secrete necrosis-inducing protein (NPP1) causing necrosis on leaf and roots of the plant, leading to death. The *NPP1* ORF contains 771 bp and encodes a 256 aa protein. The study of factors that affect *NPP1* gene expression is extremely important to better evaluate the mechanism of plant necrosis induced by *Phytophthora cinnamomi*. In order to understand its function, we evaluated the expression in different inducers media by RT-qPCR. The medium rich in glucose as carbon source were the one who showed major levels of expression.

MATERIALS AND METHODS

Genomic DNA was obtained from strain *P. cinnamomi* Pr120 as described by Cooke and Duncan (1997). Polymerase chain reaction was used to amplify a fragment of the *NPP1* gene, using degenerate oligonucleotide which were designed based on homology of previous published *Phytophthora sp.* NPP's sequences from EMBL databases. PCR was performed with 10X amplification buffer; 100ng DNA template; 0.2 mM dATP, dCTP, dGTP, and dTTP (each); 0.2μM each primer; 1.5mM MgCl2; and 1U Taq DNA polymerase (Promega), in a final reaction volume of 50 μl. Amplification consisted of: one cycle of 5 min at 94ºC, and 36 cycles of denaturing for 1 min at 94ºC; annealing for 1 min at 63ºC; and extension for 30 s at 72ºC. A final extension step of 5 min at 72ºC was done for one cycle. Full gene sequence length elucidation (1328bp) was achieved by flanking the previous known sequence with asymmetric HE-TAIL PCR using the degenerated primers R1, R2, R3 and R4 and the methodology described by Michiels *et al.*(2003), and the gene-specific primers. Three rounds of PCR were performed on a MyCycler Thermal Cycler (BIORAD), using the product of the previous PCR as a template for the next. A detailed cycler program and conditions are given in Table1.

DNA sequencing was performed using an ABI 373 automated sequencer. The open reading frame (ORF) of *P. cinnamomi NPP1* was identified by BioEdit program and submitted to EMBL databases (Accession number AM403130); Nucleotide and amino acid sequences were analyzed using FASTA programs from EMBL databases. ClustalW2 (Larkin *et al*., 2007) was used to align the *Phytophthora* genus NPP1 sequences..

Gene Expression: The expression was studied during growth in different carbon sources and was also performed a time course of *NPP1* production. To analyze gene expression was performed RT-qPCR using the *SyBr Green* method and the actin gene *Act2* as a endogenous control.

TABLE 1 - HE-TAIL PCR cycle settings and conditions*

Reaction	Number of Cycles	Thermal Settings
Primary		
	1	93°C (1min); 95°C (5min)
	5	94°C (30seg), 62°C (1min), 72°C (2min30s)
	1	94°C (30seg), 25°c ramping 72°C (3min); 72°C (2min30s)
	15	94°C (20s), 65°C (3min30s); 94°C (20s), 65°C (3min30s); 94°C (30s), 42°C (1min), 72°C (2min30s)
	1	72°C (5min), 4°C Hold
Secondary		
	12	94°C (20s); 65°C (3min30s); 94°C (20s); 65°C (3min30s); 94°C (30s); 42°C (1min); 72°C (2min30s)
	1	72°C (5min), 4°C Hold
Tertiary		
	30	94°C (30s); 42°C (1min); 72°C (2min30s)
	1	72°C (5min); 4°C Hold

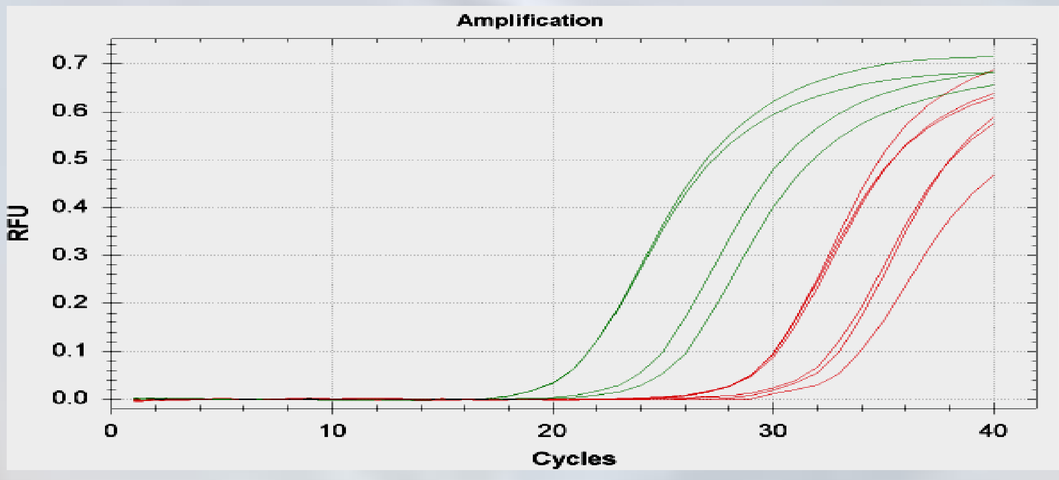

FIGURE 1 - RT-qPCR of *NPP1* gene from *P. cinnamomi*.

RESULTS AND DISCUSSION

The translated ORF of *P. cinnamomi NPP1* codifies a 256 aa protein, with a predict Mw of 29 KDa and a theoretical iso-electric point of 8,58.

In Figure 2 are shown the multiple alignment of various sequences who showed great homology with *P. cinnamomi NPP1*, including another *NPP's* of *Phytophthora* genus, and a NPP1 super-family conserved domain from oomycetes.

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P.sojae2          ---------------------------------MNLRPALLATLASFAYVSAS--VINHD 25
P.megakarya       ---------------------------------MNLR-AFVAAIA-FSGVQAA--VIDHD 23
P.sojae1          ---------------------------------MNLRRLVVAAVAFLTASHAS--VIDHD 25
P.infestans1      ------------------------------------MNLRALLFGVVASLATVTADVVMIDHD 27
P.infestans2      MLCYCILKIPLCPLSDSFLNKPHRANYLAPSSKMNLGLLFVSVLLSFVSART---TINHD 57
P.cinnamomi       ------------------------MVSAVLDSRFNVALWIFQRHPKYDVVDALR--RCQH 34
                                          :*:   ..          :      :.

P.sojae2          QVVPFTQPTPTTALQQAAVKYKPQIHISNGCHPYPAVDNNGNTSGGLNPTGSESAGCKGS 85
P.megakarya       QVVPFARPTPNTTVQSVAIQFKPQIYINDGCHPYPAVDEDGNTSGGLKPTGSQSAGCKGS 83
P.sojae1          QVVPFAQPTPTSTSQTAAVKFKPQLHITNGCHPYPAVDADGNTSGGLNPTGSSSAGCKGS 85
P.infestans1      AVQPFAQPEPTMESQKSAVKYKPQLHISYGCHPYPAVQEDGSISAGLKWAGWPNGDCKGS 87
P.infestans2      QVQPFEQIKPATDSEKSAIKYKPQLHISDGCHPYPAVQSNGSISGGLKWSGKANGDCKEP 117
P.cinnamomi       RCWEFVDPHPPR-MQRQLSSTNLSFHITDGCHPYPAVQADGSVSGGLEWSGADDGDCKGS 93
                      *    *    :    . : .::*. ********: :*. *.**: :*  ...** .

P.sojae2          GYGTQIYGRAVKYQGVYAFMYSWYMPKDE--TLTG---LGHRHDWEACVVWVDDIAASSP 140
P.megakarya       GYGSQIYGRAVEYEGVYAFMYSWCMPKDE--TLDG---LGHRHDWENCVVWLDSLDN--P 136
P.sojae1          GYGSQIYGRSTWYNGVWAIMYSWYFPKDS--PASG---FGHRHDWEHIVVWLNNPAVTSP 140
P.infestans1      ALGSQVYSRSDWYKGKWAIMYAWYFPKSR--QYVDKYRFGHRHYWSYAIVWTNNPNPDNS 145
P.infestans2      GLGSQVYSRSAWFKGKWAIMYAWYFPKGP--QFVLRFDTGHRHFWSYAILWTDDPNHDNS 175
P.cinnamomi       AWGSQVYARSAWYNNKYAIMYAWYFPKGKGRQHVHKLHSGHRHEWEFAVVWVDQPSADHS 153
                  . *:*:*.*:  ::. :*:**:* :**.          **** *.  ::* :.     .

P.sojae2          KIVALSASAHSGYNKYYPPSSSYFS-GNSAKIDYSSSYVVINHALSATSTAGETQPLIMW 199
P.megakarya       SIVALSASYHSTYLYYYPPASDYLD-GNSAKIEYSTSWIILDHSLSATSTAGETQDLIMW 195
P.sojae1          ELLAVSTSAHSGYTTYYPPSSSYLD-GNSAKIDYYN-VLLINHAFRMTSDSGETQDLIMW 198
P.infestans1      TILGVSMSSGVGYMKRSPPKSKYVVDGTTVKLDWYWSFWGSKQGLQLTEKSGETQDLITW 205
P.infestans2      TILGVSMSGSRGYVKESSPKAKYIVKGTTVKLDSYASFWMGVQALQLTKKLGETQDLITW 235
P.cinnamomi       NLLGVSMSFGPSFHKEAPVQPEHVV-GSSIKLDSYSTFWGAKQGLRVTTDIGTTQDLIQW 212
                   ::.:* *    :    .  ..:.  *.: *::          :.:  *   * ** ** *

P.sojae2          DQLTDAARRALEDTDFG------DANVPFKDANFQTKLGNAYYA 237
P.megakarya       DQLTDAARSALEDTDFG------SANVPFKEANFATKVANAYYA 233
P.sojae1          DQLTDAARTALENTDFG------DANVPFKDANFETKLANAWY- 235
P.infestans1      EQLSEEARNALSEADFDVSWAFTKAVMPLKDDIFTSRLKKAYPF 249
P.infestans2      EQLTDEACDALTEFDFESDPSRAMVVMPLGDDNFTPILKDSYPF 279
P.cinnamomi       EQLTDAARASLTETNFDIDEAVVPVEMPLKDDVFQLKLNSTYPF 256
                  :**:: *  :* : :*        . :*: :  *   : .::
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FIGURE 2 – Multiple sequence alignment of *NPP1* genes from *Phytophthora* sp.

The study of factors that affect *NPP1* gene expression is extremely important to better evaluate the mechanism of plant necrosis induced by *Phytophthora cinnamomi*. In order to understand its function, we evaluated the expression in different inducers media by RT-qPCR . The medium rich in glucose as carbon source were the one who showed major levels of expression in Figure 1.

REFERENCES

Cooke, D. & Duncan, J., 1997. Phylogenetic analysis of *Phytophthora* species based on the ITS1 and ITS2 sequences of ribossomal DNA. *Mycological Research*, 101:667-677.

Larkin, M., Blackshields, G., Brown, N., Chenna, R., McGettigan, P., McWilliam, H., Valentin, F., Wallace, I., Wilm, A., Lopez, R., Thompson, J., Gibson, T. & Higgins D., 2007. Bioinformatics, 23(21): 2947-2948.

Michiels, A., Tucker, M., Van Den Ende, W. & Van Laere, A., 2003. Chromosomal Walking of Flanking Regions From Short Known Sequences in GC-Rich Plant Genomic DNA. *Plant Molecular Biology Reporter*, 21:295-302.

Rose, J., Ham, K., Darvill, A. & Albersheim, P., 2002. Molecular cloning and characterization of glucanase inhibitor proteins: coevolution of a counterdefense mechanism by plant pathogens. *Plant Cell*, 14:1329-1345.

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